생활과학석사학위논문

Effects of Non-Traditional Extraction Methods on Extracting Bioactive Compounds from Chaga Mushroom (*Inonotus obliquus*) Compared with Hot Water Extraction

차가버섯의 유용 성분 추출에 대한 비전통적 추출 방법과 열수 추출 방법의 비교

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황 아 영

ABSTRACT

Effects of Non-Traditional Extraction Methods on Extracting Bioactive Compounds from Chaga Mushroom (*Inonotus obliquus*) Compared with Hot Water Extraction

Ah Young Hwang

Department of Food and Nutrition

The Graduate School

Seoul National University

Chaga mushroom (*Inonotus obliquus*) has been known to have antioxidant, anti-virus, anti-inflammatory, and anti-tumor effects due to its bioactive compounds such as β-glucans, triterpenoids, phenolic compounds, and melanin complexes. This mushroom has been used as a folk remedy since the 16th century and mainly consumed as extracted. Hot water extraction, which is a traditional method preparing chaga mushroom extract, has limitations requiring a large amount of solvent and long extraction time, resulting in degradation and coagulation of bioactive compounds. The objective of this study was to develop efficient methods extracting bioactive compounds in chaga mushroom, compared with hot water extraction. High

temperature and pressure, enzyme, and ultrasound extractions were used as non-traditional extraction methods. Chaga mushroom extracts were prepared under conditions determined by preliminary experiments. β-Glucans were higher in high temperature and pressure and enzyme extracts than in hot water extract. Total triterpenoids were higher in the enzyme and ultrasound extracts than in the hot water extract. Total phenolic compounds were higher in the high temperature and pressure extract than in the hot water extract. Major phenolic compounds in the chaga mushroom extracts, which were identified by HPLC, LC-MS, and GC-MS, were 3,4-dihydroxybenzaldehyde and coniferyl aldehyde, which were the highest in the high temperature and pressure and enzyme extracts, respectively. Antioxidant activities were the highest in the high temperature and pressure extract which had the highest content of phenolic compounds. In conclusion, high temperature and pressure and enzyme extractions may be efficient methods to produce chaga mushroom extracts with a large amount of bioactive compounds.

Keywords: Chaga mushroom; *Inonotus obliquus*; High temperature and pressure extraction; Enzyme extraction; Ultrasound extraction; Phenolic compounds

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INTRODUCTION

Chaga mushroom (*Inonotus obliquus*) is a fungus belonging to the family Hymenochaetaceae. It is parasitic on mature birch and mainly distributed in the countries located at 45°N-50°N latitudes (Russia, Finland, China, Japan, and so on) (Hu et al., 2009). Chaga mushroom has bioactive compounds such as β-glucans, triterpenoids, phenolic compounds, lignins, and melanin complexes to protect itself from environmental stresses such as cold, invasion of pathogen, and competition for nutrients with other microbial species (Zhong et al., 2009; Zheng et al., 2011). These bioactive compounds have been known to have antioxidant, anti-inflammatory, anti-cancer, hypoglycemic, anti-virus, and other functional activities (Zhong et al., 2009). Especially, phenolic compounds in chaga mushroom are known to have strong antioxidant activities related to therapeutic effects against diseases associated with oxidative stress such as cardiovascular diseases, diabetes, and cancer (Seo & Lee, 2010; Khatua et al., 2013).

On the other hand, chaga mushroom has traditionally been mainly consumed as extracted because it cannot be readily chewed due to its rigid cell walls, which consist of cross-linked chitin, β-glucans, and other components. Traditionally chaga mushroom extract has been prepared by heating crushed mushroom in water (Ulziijargal & Mau, 2011; Ju et al., 2010). However, this traditional extraction method requires a large amount

of solvent and long extraction time due to complex matrix of chaga mushroom. Long extraction time may lead to degradation and coagulation of extracted bioactive compounds in chaga mushroom (Rosello-Soto et al., 2016). To overcome these limitations of the conventional extraction method, different approaches such as high temperature and pressure, enzyme, and ultrasound extractions could be considered. It is well known that bioactive compounds can be destroyed at high temperature, but previous studies reported that heat treatment using high temperature and pressure efficiently extracted bioactive compounds in mushrooms by breaking bonds between bioactive compounds and cellular components (Choi et al., 2006; Seo & Lee, 2010; Jeong et al., 2004). Enzyme and ultrasound extractions are known to be economical and environmentally friendly because they could efficiently recover bioactive compounds with less energy, time, and solvent. (Xu et al., 2016; Rodrigues et al., 2017). The objective of this study was to determine bioactive compounds and antioxidant activities in chaga mushroom extracts prepared by non-traditional extraction methods such as high temperature and pressure, enzyme, and ultrasound, compared with hot water extraction.

MATERIALS AND METHODS

1. Materials

Viscozyme L was purchased from Novozymes (Bagsvaerd, Denmark). *β*-Glucan assay kit (exo-1,3-*β*-glucanase (100 U/mL) plus *β*-glucosidase (20 U/mL) ammonium sulphate suspension, glucose oxidase plus peroxidase (GOPOD) reagent enzymes, GOPOD reagent buffer, amyloglucosidase (1630 U/mL) plus invertase (500 U/mL) solution in 50% (v/v) glycerol, and D-glucose standard solution (1 mg/mL) in 0.2% (w/v) benzoic acid) was purchased from Megazyme (Bray, Ireland). HCl (37% (v/v) and 1 N), KOH, NaOH, 99.7% (v/v) glacial acetic acid, sodium bicarbonate anhydrous, 70% (v/v) perchloric acid, and formic acid were purchased from Samchun Pure Chemicals (Pyeongtaek, Korea). Vanillin, 2,5-dihydroxyterephthalic acid, and ursolic acid were from Tokyo Chemical Industry Co. (Tokyo, Japan). Folin-Ciocalteu reagent, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), pyridine, gallic acid, caffeic acid, coniferyl aldehyde, ferulic acid, 4-hydroxybenzoic acid, syringic acid, vanillic acid, 2,2′-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), potassium persulfate, and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Protocatechuic acid was from HWI Analytik (Rheinzabern, Germany). 3,4-Dihydroxybenzaldehyde was

from Wako Chemical Co. (Osaka, Japan). Methanol and acetonitrile were from JT Baker (Phillipsburg, NJ, USA). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was from Roche Diagnostics GmbH (Mannheim, Germany). Sodium acetate trihydrate was from Kanto Chemical (Tokyo, Japan). Ferric chloride anhydrous was from Duksan Pure Chemicals (Ansan, Korea).

2. Preparation of water extracts

2.1. Sample preparation

Chaga mushroom (Tyumen, Russia) was provided by DHF Company (Seoul, Korea). The outer part of chaga mushroom was discarded and the inner part was used for extraction. It was pulverized using a blender (Hanil Co., Bucheon, Korea). The chaga mushroom powder (CMP) was placed in a polyethylene pouch (Rollpack, Pyeongtaek, Korea), sealed using a vacuum packaging machine (M-6TM, Leepack Co., Incheon, Korea), and stored at 15 ℃ until extraction.

2.2. Determination of extraction conditions

Three preliminary experiments were conducted to determine appropriate extraction conditions for preparing chaga mushroom extracts. In the first preliminary experiment, different ratios of CMP to water (1:20, 1:30, 1:40, and 1:50) were compared in hot water extraction. In the second experiment,

various extraction times (1, 2, 4, and 6 h) were compared in hot water extraction. The appropriated ratio of CMP to water and extraction time determined in the hot water extraction were applied to all the extraction methods used in this study, because hot water extraction is a traditional method producing chaga mushroom extracts. The third preliminary experiment was performed in enzyme extraction to determine appropriate addition level of the enzyme, comparing 1, 3, 5, and 7% (v/w).

2.3 Preparation of chaga mushroom water extracts

Extraction procedure is shown in Fig. 1. Hot water extract (HWE): The CMP (10 g) and 400 mL water were mixed in a round-bottom flask, which was then refluxed for 2 h in a water bath (Daihan Scientific Co., Seoul, Korea) at 100 °C.

High temperature and pressure extract (HE): The CMP (10 g) and 400 mL water were mixed in a Duran's bottle, which was heated for 2 h in an autoclave (AC-11, Jeiotech, Seoul, Korea) at 121 °C.

Enzyme extract (EE): The CMP (10 g) was dispersed in 400 mL water. pH was adjusted to 4.5, which is optimum pH of Viscozyme L. The enzyme was added at 5% (v/w). The mixture was heated for 2 h, stirring at 200 rpm with an overhead stirrer (HS-30D, Daihan Scientific Co.) in the water bath at 50 °C. The enzyme was inactivated heating the extract at 90 °C for 10 min.

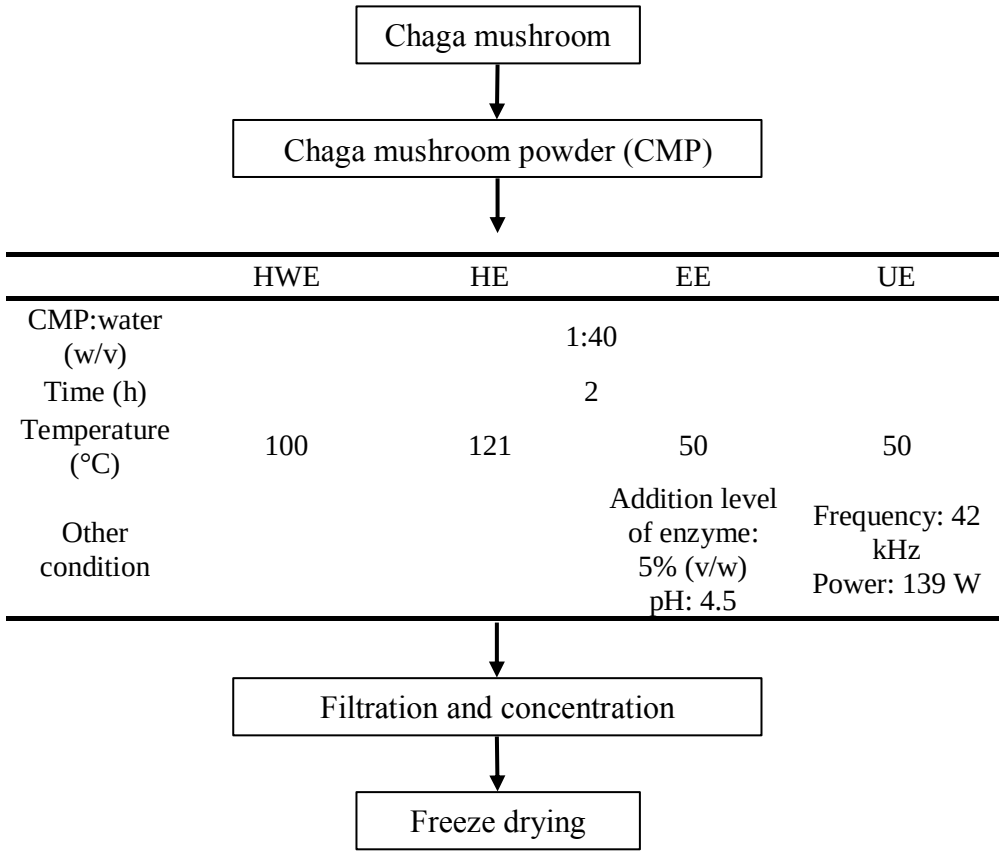

Figure 1. Flow chart of preparing chaga mushroom extracts. HWE, hot water extract; HE, high temperature and pressure extract; EE, enzyme extract; and UE, ultrasound extract.

Ultrasound extract (UE): The CMP (10 g) and 400 mL water were placed in the Duran's bottle, followed by heating for 2 h in an ultrasonic bath (5510E-DTH, 139 W, 42 kHz, Bransonic, Danbury, CT, USA) at 50 °C.

Each of the extracts was filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England). The filtrate was concentrated using a rotary evaporator (A-10005, Eyela Co., Tokyo, Japan) at 50 °C under vacuum. The concentrated extract was freeze-dried and then stored at -20 °C until further analysis.

3. Determination of glucans

Content of β-glucans was determined according to protocol of β-glucan assay kit. Briefly, to measure total glucan and glucose content, the extract powder (100 mg) was dispersed in 1.5 mL 37% HCl solution, followed by incubation for 45 min in the water bath at 30 °C. Water (10 mL) was added, followed by heating at 100 °C for 2 h to hydrolyze α-glucans to glucose. The hydrolyzed solution was cooled down to room temperature and neutralized adding 10 mL 2 M KOH. The neutralized sample was centrifuged at 1500 × g for 10 min and supernatant was collected. The supernatant (0.1 mL) was mixed with 0.1 mL exo-1,3-β-glucanase plus β-glucosidase mixture, followed by incubation at 40 °C for 60 min to hydrolyze β-glucans to glucose. GOPOD reagent (3 mL) was added to the hydrolyzed sample, followed by incubation at 40 °C for 20 min. Absorbance

was measured at 510 nm against reagent blank using a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA).

To determine α-glucan and glucose content, the extract powder (100 mg) was incubated with 2 mL 2 M KOH for 20 min in an ice bath, followed by neutralization with 8 mL 1.2 M sodium acetate buffer (pH 3.8). Mixture of amyloglucosidase plus invertase (0.2 mL) was added to the neutralized sample to hydrolyze α-glucans to glucose, followed by incubation at 40 °C for 30 min. The hydrolyzed sample was centrifuged at 1500 × g for 10 min and supernatant was collected. The supernatant (0.1 mL) was mixed with 3 mL GOPOD reagent, followed by incubation at 40 °C for 20 min. Absorbance was measured at 510 nm against reagent blank.

Contents of total glucans and glucose and α-glucans and glucose were calculated comparing with D-glucose standard. β-Glucan content was calculated by subtracting the α-glucan and glucose content from the total glucan and glucose content.

4. Determination of total triterpenoids

Total triterpenoids were determined as described by Chen et al. (2007) with some modifications. The extract powder was dispersed in 100% methanol at 5 mg/mL, followed by filtering through 0.2 μm nylon syringe filter (Whatman International Ltd.). One hundred μL of the filtrate, 0.15 mL 5% (w/v) vanillin-acetic acid solution, and 0.5 mL 70% perchloric acid were

mixed and heated at 60 ℃ for 45 min. It was left at room temperature for 3 min and mixed with 2.25 mL acetic acid. Absorbance was measured at 548 nm. Total triterpenoids were expressed as ursolic acid equivalent (UAE).

5. Determination of total phenolic compounds

Total phenolic compounds were determined by a modified method of Singleton et al. (1999). The extract powder (2 mg) was dispersed in 1 mL 50% methanol. The methanol dispersion (20 μL) was mixed with water (1.58 mL) and Folin-Ciocalteu reagent (100 μL). The mixture was vortexed, left for 3 min at room temperature, and mixed with 300 μL 20% (w/v) sodium bicarbonate solution, followed by incubation at 40 ℃ for 30 min. Absorbance was measured at 765 nm. Content of total phenolic compounds was expressed as gallic acid equivalent (GAE).

6. Identification of phenolic compounds

Phenolic compounds in the chaga mushroom extracts were identified by high performance liquid chromatography (HPLC) and mass spectrometry (MS) using an UltiMate 3000 RS HPLC system coupled with an LTQ XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) with electrospray ionization (ESI). The chaga mushroom extract powder (100 mg) dispersed in 1 mL methanol was sonicated for 20 min, followed by filtering with 0.2 μm nylon syringe filter. The filtrate was transferred into a

glass vial (Agilent, Santa Clara, CA, USA). The filtrate (5 μL) was injected into the HPLC system with a U-VDSpher PUR C18-E (100 × 2.0 mm i.d., 1.8 μm particle size, VDS Optilab, Berlin, Germany). Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was as follows: 0-6 min, 92-88% A; 6-15 min, 88-82.5% A; 15-25 min, 82.5-73% A; 25-26 min, 73-0% A; 26-27 min, 0% A; 27-27.5 min, 92% A; and 27.5-35 min, 92% A. Flow rate was 0.3 mL/min. The mass spectrometer was operated in both positive and negative modes with source voltages of 3.5 kV and 2.7 kV, respectively. Capillary temperature was 300 °C. MS data were obtained in a range of 100-1000 *m/z*.

GC-MS analysis was performed by method of Ju et al. (2010). The chaga mushroom extract powder was dispersed in methanol at 100 mg/mL. The methanol dispersion was sonicated for 20 min, followed by filtering through 0.2 μm nylon syringe filter. After the solvent in the filtrate was evaporated under nitrogen stream, BSTFA containing 1% TMCS (300 μL) and pyridine (200 μL) were added, followed by incubation at 60 °C for 30 min to silylate. The silylated sample (1 μL) was injected into a GCMS-QP2010 Plus system (Shimadzu Co., Kyoto, Japan) with a DB-5 capillary column (30 m × 0.25 mm i.d., 0.25 μm thickness, J&W Scientific, Folsom, CA, USA). Column temperature was initially held at 100 °C for 2 min, then raised to 270 °C at 5 °C /min, and held for 6 min. Carrier gas was helium and flow rate was 1.2 mL/min. Injector temperature was 250 °C and interface temperature was

230 °C. Ion source temperature was 230 °C and electron energy was 70 eV. Mass spectra were scanned from 100 to 1000 *m/z* and matched with NIST (National Institute of Standards and Technology) library for identification of phenolic compounds.

7. Quantification of phenolic compounds

The extract powder was dispersed in 50% methanol (20 mg/mL), followed by filtering with 0.2 μm nylon syringe filter. The filtrate (20 μL) was loaded on a Waters 2695 HPLC system equipped with a Zorbax Eclipse Plus C18 column (250 × 4.6 mm i.d., 5 μm particle size, Agilent) and a diode array detector. Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was as follows: 0-6 min, 92-88% A; 6-15 min, 88-82.6% A; 15-27 min, 82.6-73% A; 27-27.1 min, 73-92% A; and 27.1-35 min, 92% A. Column oven was 30 °C and flow rate was 1 mL/min. Absorbance was measured at 280 nm. Phenolic compounds in the chaga mushroom extracts were quantified by their corresponding standards (gallic acid, protocatechuic acid, 3,4-dihydroxybenzaldehyde, caffeic acid, syringic acid, ferulic acid, and coniferyl aldehyde).

8. Antioxidant activities

8.1. DPPH radical scavenging activity

DPPH free radical scavenging activity was analyzed by a modified

method from Brand-Williams et al. (1995). The extract powder (0.1 mg) was dispersed in 1 mL 50% methanol. The methanol dispersion (50 μL) was mixed with 100 μL 0.2 mM DPPH methanol solution, followed by incubation at room temperature for 30 min in the dark. Absorbance was measured at 517 nm. The result was expressed as Trolox equivalent antioxidant capacity (TEAC).

8.2. ABTS radical scavenging activity

ABTS radical scavenging activity was determined using method developed by Re et al. (1999). Briefly, 7 mM ABTS solution and 2.45 mM potassium persulfate solution were mixed at a ratio of 1:1, followed by incubation for 16 h in the dark at room temperature to produce ABTS radical cation solution ($ABTS^{\cdot+}$), which was diluted with water to obtain an absorbance of 0.7 ± 0.03 at 734 nm. The extract powder was dispersed in 50% methanol at 0.1 mg/mL. The diluted $ABTS^{\cdot+}$ solution (1 mL) was added to the methanol dispersion (10 μL). Absorbance was measured at 734 nm. The result was expressed as TEAC.

8.3. Ferric reducing antioxidant power (FRAP)

FRAP assay was performed according to method of Benzie and Strain (1996). To make FRAP working solution, 300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ solution

were mixed at a ratio of 10:1:1 prior to use and warmed at 37 ℃ for 15 min. The extract powder was dispersed in 50% methanol at 2 mg/mL. The methanol dispersion (20 μL) was pipetted into a 96-well plate. The pre-warmed FRAP working solution (180 μL) was added to each well of the 96-well plate, followed by incubation at 37 ℃ for 15 min. Absorbance was measured at 593 nm. The result was expressed as TEAC.

9. Statistical analysis

All experiments were conducted in triplicate except the preliminary experiments and MS analysis, which were conducted once. Analysis of one-way analysis of variance (ANOVA) and Duncan's multiple range test (p<0.05) were conducted using a SPSS program (version 23.0, SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

1. Yields of chaga mushroom extracts

Yield of the chaga mushroom extracts prepared by hot water increased when the ratio of CMP to water decreased from 1:20 to 1:40, but there was no big difference in extraction yield between 1:40 and 1:50 (Fig. 2A). The extraction yield by hot water increased with extraction time up to 2 h from 20.5% at 1 h to 22.5% at 2 h, but there was no big difference in extraction yield after 2 h (Fig. 2B). Therefore, appropriate ratio of CMP to water and extraction time for preparing chaga mushroom extract by hot water were 1:40 and 2 h, respectively. This extraction condition was applied to all the extraction methods in this study. Extraction yield by the enzyme with its addition levels of 1, 3, 5, and 7% (v/w) increased with the level from 1% to 5%, but it did not change at level of 7% (Fig. 2C). Therefore, addition level of the enzyme for the EE was set to 5%.

2. Contents of bioactive compounds

2.1. Contents of glucans

β-Glucan content was the highest in the HE (Table 1). This is similar to the result of Alzorqi et al. (2017), reporting that *β*-glucan content was higher in *Ganoderma lucidum* treated at 99.6 ℃ than at 76 ℃, probably because *β*-

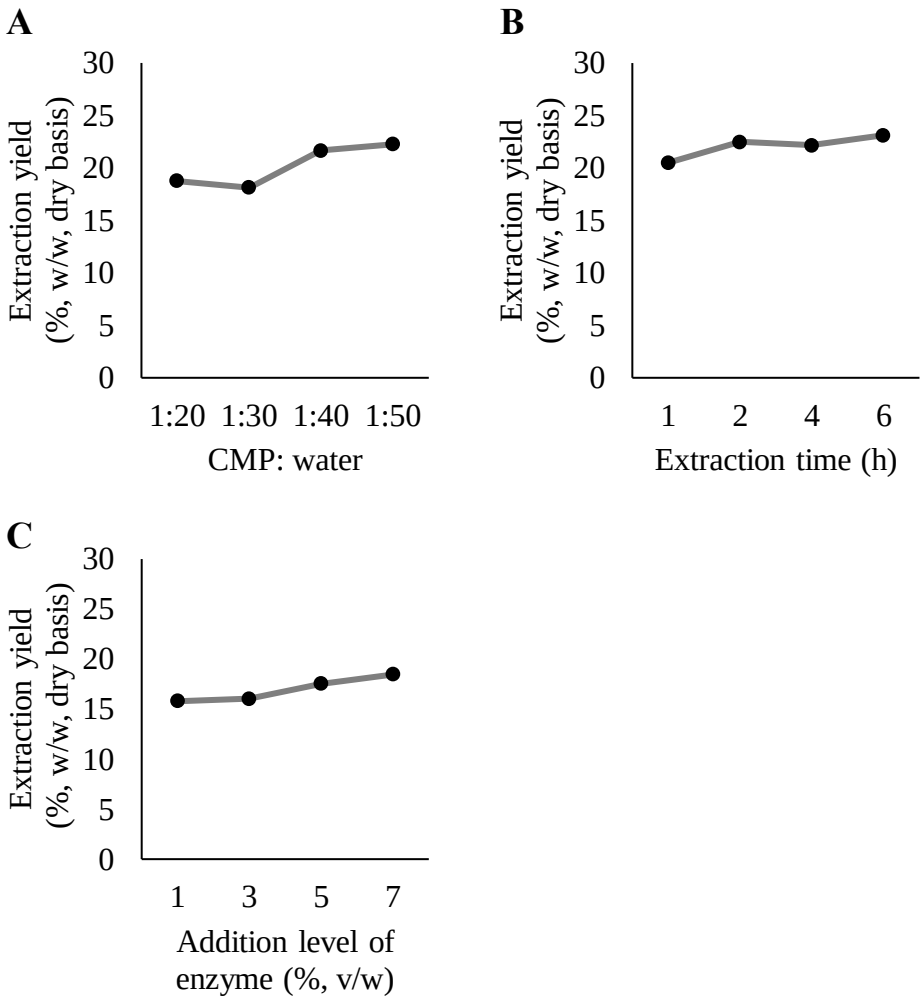

Figure 2. Effects of ratio of chaga mushroom powder (CMP) to water (A) and extraction time (B) in hot water extraction and addition level of enzyme in enzyme extraction (C) on extraction yield of chaga mushroom.

glucans might be more soluble at a higher temperature. The β-glucan content was significantly ($p<0.05$) higher in the EE than in the HWE. Rosello-Sato et al. (2016) reported that enzymes with chitinase or glucanase activity could intensify extraction of compounds in mushrooms by breaking bonds between cell wall components (β-glucans, α-glucans, chitin, proteins, and so on) of mushrooms more efficiently than hot water extraction. Fu et al. (2010) reported that ultrasound-assisted extraction effectively extracted β-glucans in chaga mushroom. However, in the present study, the content of β-glucans was the lowest in the UE, suggesting ultrasonic temperature might not be suitable to extract β-glucans. Cheung et al. (2012) reported that polysaccharide yields of *Grifola frondosa*, *Coriolus versicolor*, and *Lentinus edodes* extracts prepared by ultrasound-assisted extraction at 50 ℃ were lower than those prepared by hot water extraction at 100 ℃; however, polysaccharide yields of *C. versicolor* and *L. edodes* extracts prepared by the ultrasound-assisted extraction at 80 ℃ were higher than those prepared by the hot water extraction.

2.2. Content of triterpenoids

The UE and EE were significantly ($p<0.05$) higher than the HWE and HE in total triterpenoids (Table 1). Previous studies reported that cavitation phenomenon induced by ultrasound or enzymatic hydrolysis could efficiently rupture mushroom cell walls and break bonds between

Table 1. Glucans, triterpenoids, and phenolic compounds in chaga mushroom extracts

	Total glucans and glucose (%, w/w, dry basis)	α-Glucans and glucose (%, w/w, dry basis)	β-Glucans (%, w/w, dry basis)	Total triterpenoids (mg UAE/g)	Total phenolic compounds (mg GAE/g)
HWE	3.81±0.34^{b}	2.66±0.42^{ab}	1.15±0.12^{c}	8.90±0.30^{c}	166±8.58^{b}
HE	5.88±0.06^{a}	2.71±0.09^{ab}	3.17±0.04^{a}	7.03±0.76^{d}	204±8.91^{a}
EE	5.86±0.34^{a}	3.09±0.07^{a}	2.77±0.27^{b}	24.3±0.05^{a}	72.0±1.16^{d}
UE	3.02±0.05^{c}	2.35±0.09^{b}	0.68±0.13^{d}	16.2±0.13^{b}	99.9±2.25^{c}

HWE, hot water extract; HE, high temperature and pressure extract; EE, enzyme extract; UE, ultrasound extract; UAE, ursolic acid equivalent; and GAE, gallic acid equivalent.

Values are means ± standard deviations (n=3).

$^{a-d}$ Different superscripts in the same columns indicate significant differences (p<0.05).

triterpenoids and cellular components (Tohtahon et al., 2017; Song et al., 2016), facilitating triterpenoids in mushroom to be transferred to extraction solvent. The content of triterpenoids in the HE was significantly (p<0.05) lower than that in the HWE, indicating that triterpenoids might be destroyed by high temperature. Wu et al. (2001) also reported that some of triterpenoids were destroyed at 118 °C.

2.3. Content of phenolic compounds

Content of total phenolic compounds was the highest in the HE, followed by the HWE, EE, and UE (Table 1). Choi et al. (2006) reported that phenolic compounds in shiitake mushroom treated at 121 °C for 30 min were higher than at 100 °C for 30 min. Seo and Lee (2010) also reported that phenolic compounds in chaga mushroom extracts produced by supercritical water extraction increased with temperature, indicating that temperature was critical in extracting phenolic compounds from chaga mushroom.

3. Identification of phenolic compounds

LC-MS spectra of 2,5-dihydroxyterephthalic acid, 3,4-dihydroxybenzaldehyde, and coniferyl aldehyde are shown in Fig. 3. GC-MS spectra of gallic, protocatechuic, caffeic, syringic, and vanillic acids are shown in Fig. 4. Mass information is shown in Table 2. Structures of

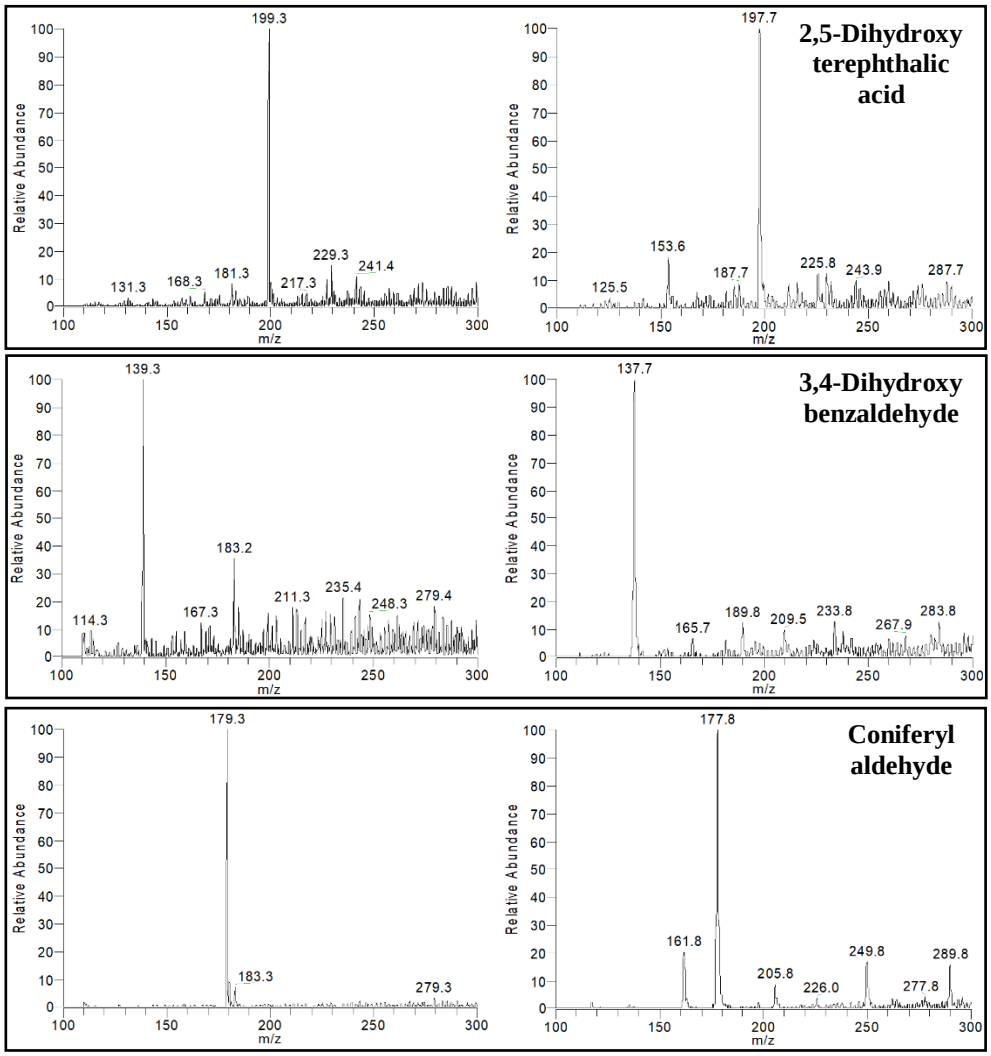

Figure 3. LC-MS spectra of phenolic compounds in chaga mushroom extracts with positive (left) and negative (right) ion modes.

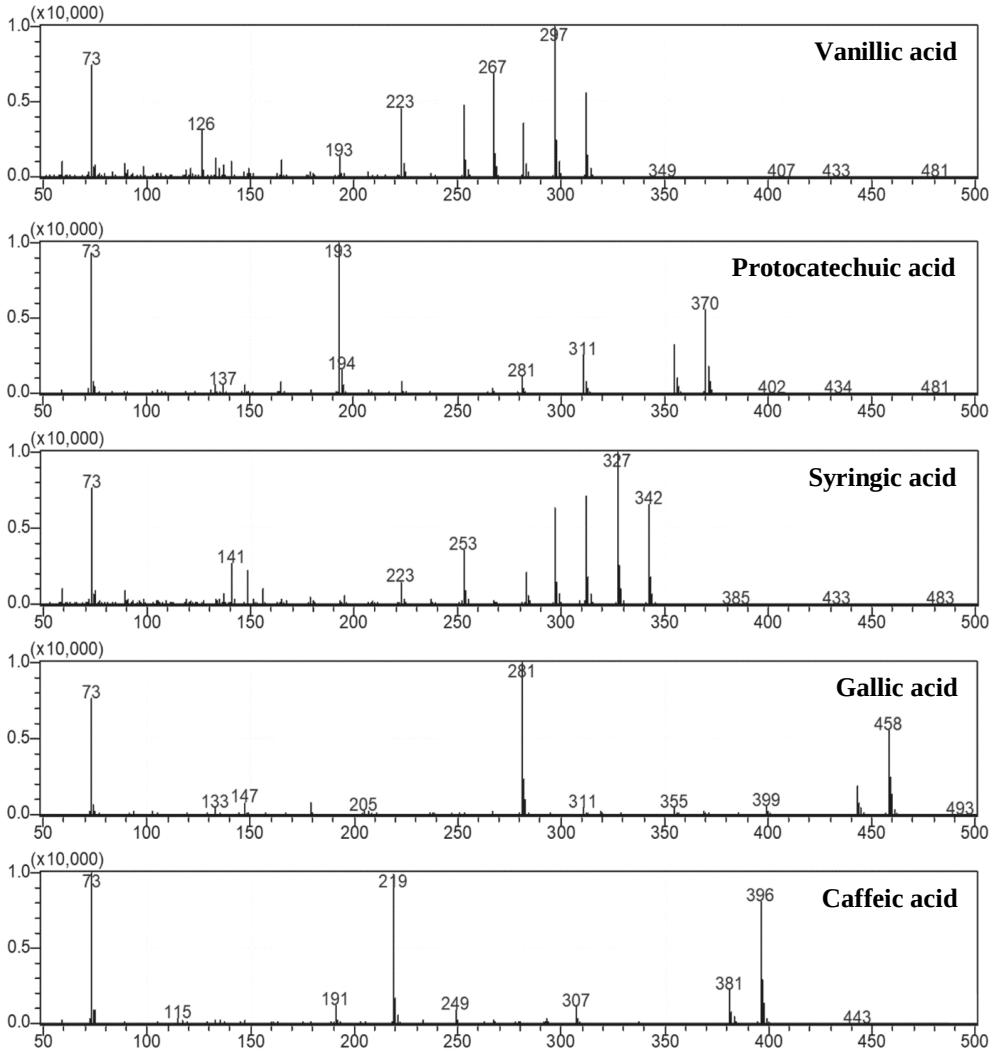

Figure 4. GC-MS spectra of phenolic compounds in chaga mushroom extracts.

Table 2. Characterization of phenolic compounds in chaga mushroom extracts

	Phenolic compound	Molecular weight (g/mol)	*m/z* (intensity, %)	Reference
LC-MS	2,5-Dihydroxyterephthalic acid	198.13	199.3 (100) $[M+H]^+$, 197.7 (100) $[M-H]^-$	Ju et al. (2010)
	3,4-Dihydroxybenzaldehyde	138.12	139.3 (100) $[M+H]^+$, 137.7 (100) $[M-H]^-$	Hwang et al. (2016)
	Coniferyl aldehyde	178.18	179.3 (100) $[M+H]^+$, 177.8 (100) $[M-H]^-$	Garcia et al. (2012)
GC-MS	Vanillic acid	312 (di-TMS)	297 (100), 267, 223, 126, 73	Ju et al. (2010)
	Protocatechuic acid	370 (tri-TMS)	370, 311, 193 (100), 73	Ju et al. (2010)
	Syringic acid	342 (di-TMS)	342, 327 (100), 253, 141, 73	Ju et al. (2010)
	Gallic acid	458 (tetra-TMS)	458, 281 (100), 73	Zheng et al. (2008)
	Caffeic acid	396 (tri-TMS)	396, 219 (100), 73	Nakajima et al. (2007)

TMS, trimethylsily.

phenolic compounds in chaga mushroom extracts identified by LC-MS and GC-MS are shown in Fig. 5. The phenolic compounds in the chaga mushroom extracts identified in the present study were also reported in previous studies except coniferyl aldehyde (Ju et al., 2010; Hwang et al., 2016; Zheng et al., 2008; Nakajima, Sato, & Konishi, 2007). In a previous study, the compound with *m/z* 179 $[M+H]^+$ identified as coniferyl aldehyde in the present study was determined as 4-(3,4-dihydroxyphenyl)but-3-en-2-one (Hwang, Lee, & Yun, 2016). However, Soares et al. (2012) reported that coniferyl aldehyde could be produced by degradation of lignin. Niu et al. (2016) reported that chaga mushroom water extract has lignin-carbohydrate complexes. Therefore, coniferyl aldehyde might exist in the chaga mushroom extracts of the present study.

The compound which had *m/z* 137 $[M-H]^-$ in this study was identified as 3,4- dihydroxybenzaldehyde (Fig. 3). However, 4-hydroxybenzoic acid may also have *m/z* 137 $[M-H]^-$ and was reported to be present in chaga mushroom (Kim et al., 2008). To exactly determine this compound which had *m/z* 137 $[M-H]^-$, HPLC retention times and UV spectra of 4-hydroxybenzoic acid and 3,4-dihydroxybenzaldehyde standards were compared with those of the chaga mushroom extracts. Retention times of 4-hydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, and the compound which had *m/z* 137 $[M-H]^-$ in the chaga mushroom extracts were 11.345, 10.459, and 10.631 min, respectively, and maximum absorption wavelengths were

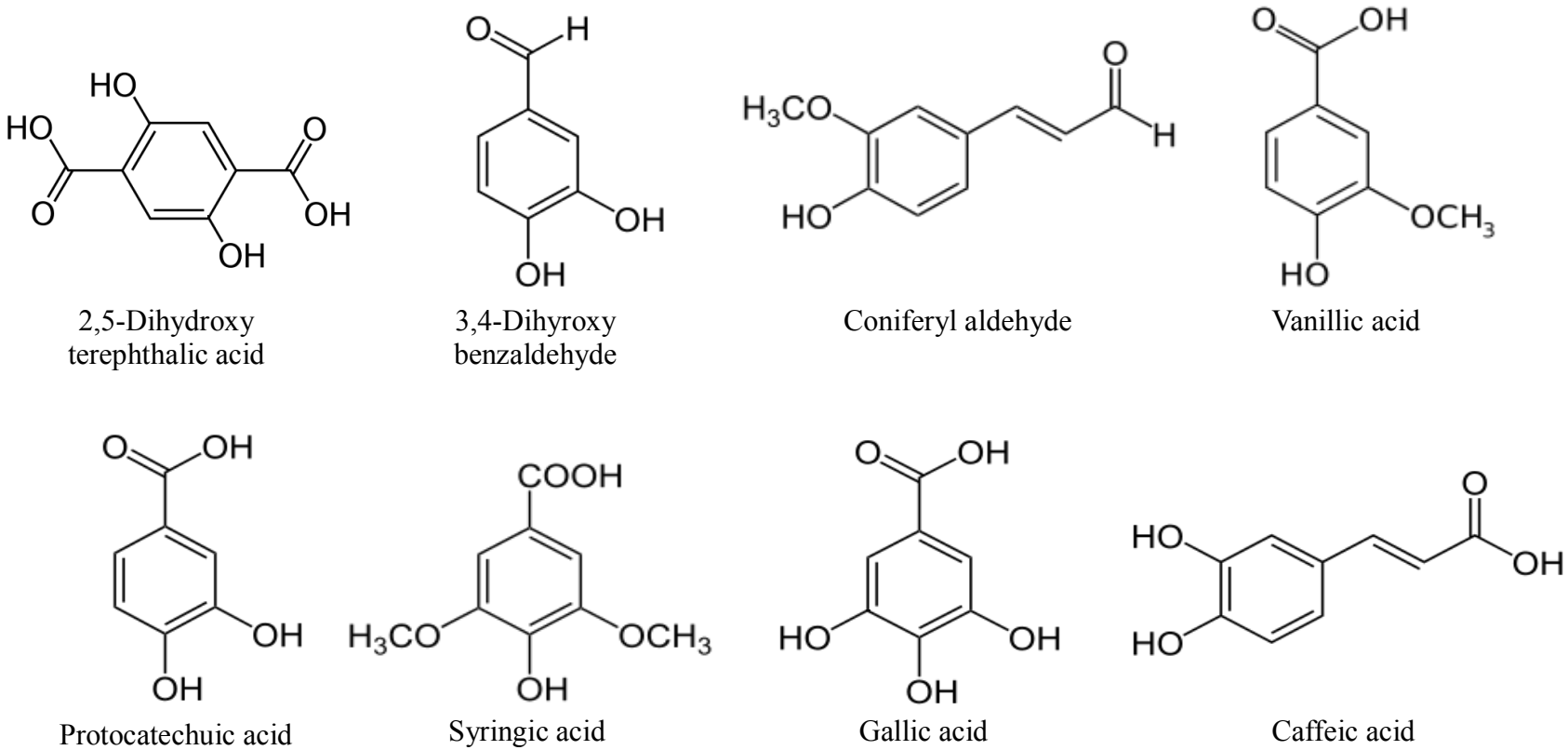

Figure 5. Structures of phenolic compounds in chaga mushroom extracts identified by LC-MS and GC-MS.

217 and 243 nm; 230 and 280 nm; and 230 and 279 nm, respectively. Therefore, the compound which had *m/z* 139 $[M-H]^-$ in the chaga mushroom extracts should be 3,4-dihydroxybenzaldehyde.

Retention times of phenolic compounds in the chaga mushroom extracts identified by LC-MS and GC-MS were compared with those of their corresponding standards using HPLC (Fig. 6). 2,5-Dihydroxyterephthalic and vanillic acids in the chaga mushroom extracts were detected by the LC-MS and GC-MS, but not on the HPLC. Peak 6 of the HPLC was presumed to be ferulic acid comparing the retention time to that of its corresponding standard.

4. Quantification of phenolic compounds

Seven phenolic compounds (gallic acid, protocatechuic acid, 3,4-dihydroxybenzaldehyde, caffeic acid, syringic acid, ferulic acid, and coniferyl aldehyde) in the chaga mushroom extracts were quantified using their corresponding standards (Table 3). Two unknown HPLC peaks of phenolic compounds in the chaga mushroom extracts were quantified as GAE.

Gallic and caffeic acids were the highest in the UE. This might be because gallic and caffeic acids were known to be destroyed by heat treatment (Boles et al., 1988; Kim, 2009). Chaga mushroom has been known to have inhibitory

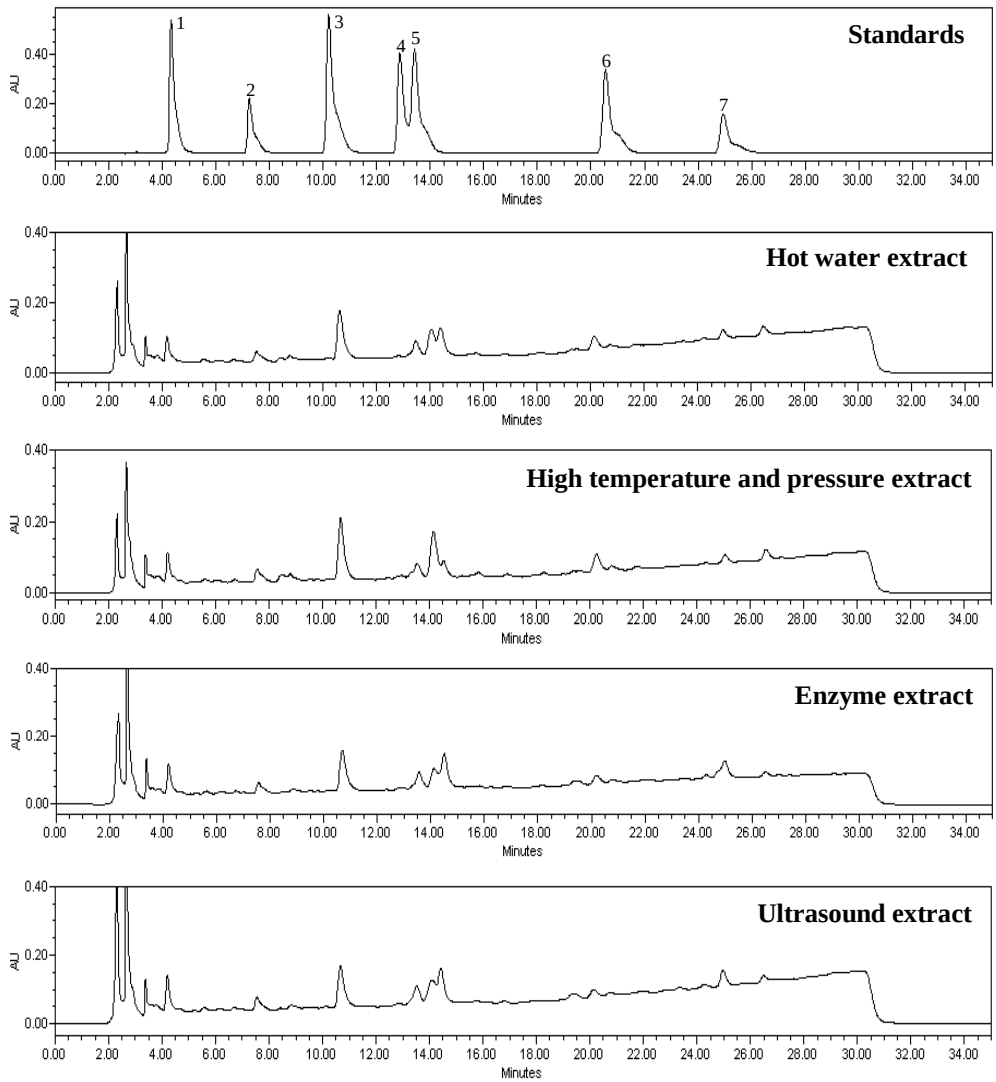

Figure 6. HPLC chromatograms of phenolic compounds in standard solution and chaga mushroom extracts (λ=280 nm). 1, gallic acid; 2, protocatechuic acid; 3, 3,4-dihydroxybenzaldehyde; 4, caffeic acid; 5, syringic acid; 6, ferulic acid; and 7, coniferyl aldehyde.

Table 3. Phenolic compounds (mg/g) in chaga mushroom extracts

	Gallic acid	Protocatechuic acid	3,4-Dihydroxy benzaldehyde	Caffeic acid	Syringic acid	Ferulic acid[1]	Coniferyl Aldehyde	Unknown A[2]	Unknown B[2]	Total
HWE	0.83±0.03^{d}	0.90±0.03^{c}	1.45±0.07^{b}	0.80±0.02^{b}	0.93±0.02^{b}	0.61±0.04^{b}	0.65±0.03^{c}	1.40±0.02^{b}	0.37±0.01^{b}	7.95±0.26^{b}
HE	0.91±0.01^{c}	1.07±0.01^{a}	1.67±0.02^{a}	0.72±0.02^{c}	1.54±0.03^{a}	0.77±0.04^{a}	0.52±0.01^{d}	0.66±0.01^{c}	0.44±0.00^{a}	8.31±0.08^{b}
EE	0.95±0.01^{b}	0.98±0.02^{b}	1.32±0.05^{c}	0.85±0.02^{b}	0.68±0.01^{c}	0.42±0.01^{c}	1.70±0.03^{a}	1.91±0.17^{a}	0.30±0.02^{c}	9.10±0.40^{a}
UE	1.05±0.00^{a}	1.11±0.04^{a}	1.30±0.05^{c}	0.99±0.07^{a}	0.90±0.07^{b}	0.42±0.03^{c}	1.11±0.08^{b}	1.93±0.18^{a}	0.21±0.00^{d}	9.02±0.25^{a}

HWE, hot water extract; HE, high temperature and pressure extract; EE, enzyme extract; and UE, ultrasound extract.

Values are means ± standard deviations (n=3).

[1]Presumed.

[2]Unknown A and B are expressed as gallic acid equivalent.

[a-d] Different superscripts in the same columns indicate significant differences (p<0.05).

effect on cancer because it has various bioactive compounds including phenolic compounds, especially gallic and caffeic acids (Balandaykin et al., 2015). Kuriyama et al. (2013) reported that gallic and caffeic acids among phenolic compounds in chaga mushroom had efficient anti-cancer activity by inhibiting topoisomerase II. Huang et al. (2009) and Rocha et al. (2012) also reported inhibitory effects of gallic and caffeic acids on cancer.

Syringic and ferulic acids were the highest in the HE. Ju et al. (2010) reported that contents of protocatechuic and syringic acids in chaga mushroom steam-treated at 121 °C for 3 h were higher than those in untreated chaga mushroom. They also reported that bound phenolic compounds in steam-treated chaga mushroom decreased, while free phenolic compounds increased. Xu et al. (2007) reported that free ferulic acid in citrus peel extracts prepared at 120 °C increased by increasing heating time with decreasing ester and glycoside ferulic acids. These previous studies suggest that syringic, ferulic, and protocatechuic acids present as some of the insoluble bound forms in chaga mushroom might be converted into soluble free forms by heat treatment.

Coniferyl aldehyde was significantly ($p<0.05$) higher in the EE and UE than in the HWE and HE, while 3,4-dihydroxybenzaldehyde was significantly ($p<0.05$) higher in the HWE and HE than in the EE and UE.

Total content of phenolic compounds quantified by the HPLC was significantly ($p<0.05$) higher in the EE and UE than in the HWE and HE.

This result was different from the result obtained by Folin-Ciocalteu reagent. Total amount of phenolic compounds detected by the HPLC was less than that quantified by Folin-Ciocalteu reagent. This might result from the fact that Folin-Ciocalteu reagent can react with phenolic compounds as well as with other antioxidant substances such as polysaccharides, proteins, and melanin complexes (Ju et al., 2010).

5. Antioxidant activities

The HE exhibited the highest antioxidant activities, followed by the HWE, EE, and UE (Table 4), which were highly correlated with the content of total phenolic compounds. However, this result was not consistent with the sum of phenolic compounds measured by the HPLC. Nakajima et al. (2007) and Hu et al. (2009) reported that major substances related to antioxidant activities in chaga mushroom water extracts were polysaccharide-protein and melanin complexes rather than small phenolic compounds. Hu et al. (2009) also reported that polysaccharide-protein complexes were higher in chaga mushroom extract treated at 80 ℃ than in that treated at 50 ℃. Zou et al. (2010) reported that extraction of melanins in *Auricularia auricula* was accelerated by increasing extraction temperature.

Table 4. Antioxidant activities (μM TEAC/mg) of chaga mushroom extracts

	DPPH	ABTS	FRAP
HWE	722±39.1[b]	828±19.9[b]	779±17.5[b]
HE	872±22.8[a]	972±23.7[a]	995±34.2[a]
EE	425±11.0[c]	538±23.6[c]	404±15.6[c]
UE	359±12.3[d]	383±17.9[d]	316±14.2[d]

TEAC, Trolox equivalent antioxidant capacity; HWE, hot water extract; HE, high temperature and pressure extract; EE, enzyme extract; and UE, ultrasound extract.

Values are means ± standard deviations (n=3).

[a-d] Different superscripts in the same columns indicate significant differences (p<0.05).

CONCLUSION

β-Glucans were higher in the HE and EE than in the HWE. Total phenolic compounds and antioxidant activities were higher in the HE than in the HWE. Total triterpenoids were higher in the EE and UE than in the HWE. In conclusion, β-glucans, triterpenoids, and phenolic compounds in chaga mushroom were more efficiently extracted in the HE, EE, and UE than in the HWE. Since contents of β-glucans, triterpenoids, and phenolic compounds and antioxidant activities in the chaga mushroom extracts were affected by extraction methods, a specific extraction method corresponding to a group or each of target compounds may be needed to maximize extraction. Further studies on preparing chaga mushroom extracts by combining two or more extraction methods and on effects of chaga mushroom extracts prepared by various extraction methods on anti-cancer, anti-inflammatory, anti-virus, and other functional activities may also be needed.

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국문초록

차가버섯의 유용 성분 추출에 대한 비전통적 추출 방법과 열수 추출 방법의 비교

황아영

식품영양학과

서울대학교 대학원

차가버섯은 베타글루칸, 트리터페노이드, 페놀화합물 등과 같은 다양한 유용 성분을 함유하고 있으며, 이러한 물질들의 항암, 항산화, 항염증, 항당뇨 등의 효과가 있다고 알려져 16세기부터 약용버섯으로 사용되어 왔다. 차가버섯은 차가버섯 가루를 뜨거운 물에 끓이는 전통적인 방법으로 추출하여 섭취해왔다. 이 열수 추출 방법은 많은 양의 물을 필요로 하며 추출 시간이 많이 소요되기 때문에 차가버섯에 존재하는 유용 성분들이 분해되거나 응집될 수 있는 단점이 있다. 따라서 본 연구에서는 차가버섯 추출물을 제조하는 효율적인 방법을 탐색하기 위하여 열수 추출법으로 제조한 차가버섯 추출물과 비전통적인 추출 방법인 고온고압, 초음파, 효

소 추출법으로 제조한 차가버섯 추출물의 유용 성분 함량과 항산화능을 비교하였다. 추출물 제조 조건은 예비실험을 통해 차가버섯과 물의 비율을 1:40으로, 추출 시간은 2시간으로 설정하였으며, 그 이외의 조건들은 각 추출 방법에서 가장 일반적으로 사용되는 조건을 사용하였다. 베타글루칸과 페놀 화합물은 고온고압 추출물에 가장 많이 함유되어 있었으며, 트리터페노이드는 효소 추출물에 가장 많이 함유되어 있었다. 베타글루칸은 열수 추출물보다 효소 추출물에 더 많이 함유되어 있었다. 트리터페노이드는 열수 추출물보다 초음파 추출물에 더 많이 함유되어 있었다. 이를 통하여 비전통적인 추출 방법들이 열수 추출 방법보다 차가버섯에 존재하는 유용 성분들을 더 효율적으로 추출함을 알 수 있었다. 페놀 화합물을 분석하기 위하여 LC-MS, GC-MS, LC를 이용하였다. 차가버섯 추출물에 함유되어 있는 페놀 화합물은 vanillic acid, 2,5-dihydroxyterephthalic acid, gallic acid, protocatechuic acid, 3,4-dihydroxybenzaldehyde, caffeic acid, syringic acid, ferulic acid(추정), coniferyl aldehyde 등이었다. 이 페놀 화합물 중 3,4-dihydroxybenzaldehyde와 coniferyl aldehyde가 가장 많이 함유되어 있었으며, 전자는 고온고압 추출물에, 후자는 효소 추출물에 가장 많이 함유되어 있었다. 항산화능은 총 페놀 화합물이 가장 많이 함유되어 있었던 고온고압 추출물이 가장 높았다. 따라

서 고온고압 추출 방법을 통하여 차가버섯에 존재하는 유용 성분들을 가장 효율적으로 추출할 수 있었고, 이 추출물의 항산화능도 가장 높았다.

주요어: 차가버섯, 고온고압, 초음파, 효소, 페놀 화합물

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